



HOKKAIDO UNIVERSITY

Title	Polyhydroxyalkanoate production by a novel bacterium <i>Massilia</i> sp UMI-21 isolated from seaweed, and molecular cloning of its polyhydroxyalkanoate synthase gene
Author(s)	Han, Xuerong; Satoh, Yasuharu; Kuriki, Yumi et al.
Citation	Journal of bioscience and bioengineering, 118(5), 514-519 https://doi.org/10.1016/j.jbiosc.2014.04.022
Issue Date	2014-11
Doc URL	https://hdl.handle.net/2115/57954
Type	journal article
File Information	manuscript_UMI021_JBB(140423).pdf



Polyhydroxyalkanoate production by a novel bacterium *Massilia* sp. UMI-21 isolated from seaweed, and molecular cloning of its polyhydroxyalkanoate synthase gene

(Running title: PHA production by a bacterium isolated from seaweed)

Xuerong Han,¹ Yasuharu Satoh,¹ Yumi Kuriki,¹ Teruyuki Seino,² Shinji Fujita,³ Takanori Suda,¹
Takanori Kobayashi,⁴ Kenji Tajima,^{1,*}

Faculty of Engineering, Hokkaido University, N13W8, Kita-ku, Sapporo 060-8628, Japan,¹ Hakodate National College of Technology, 14-1 Tokura-chou, Hakodate 042-8501, Japan,² Graduate School of Fisheries Science and Environmental Studies, Nagasaki University, 1-4 Bunkyo-chou, Nagasaki 852-8521, Japan,³ Hokkaido Industrial Technology Center, 379 Kikyuu-chou, Hakodate, 041-0801, Japan⁴

Key words: polyhydroxyalkanoate (PHA), seaweed, green algae, *Ulva*, *pha* locus, ClassI, *Massilia*

*Corresponding author. e-mail: ktajima@eng.hokudai.ac.jp

phone/fax: +81-(0)11-706-6607

ABSTRACT

We successfully isolated one microorganism (UMI-21) from *Ulva*, a green algae that contains starch. The strain UMI-21 can produce polyhydroxyalkanoate (PHA) from starch, maltotriose, or maltose as a sole carbon source. Taxonomic studies and 16S rDNA sequence analysis revealed that strain UMI-21 was phylogenetically related to species of the genus *Massilia*. The PHA content under the cultivation condition using a 10-L jar fermenter was 45.5% (w/w). This value was higher than that obtained after cultivation in a flask, suggesting the possibility of large-scale PHA production by UMI-21 from starch. A major issue for the industrial production of microbial PHAs is the very high production cost. Starch is a relatively inexpensive substrate that is also found in abundant seaweeds such as *Ulva*. Therefore, the strain isolated in this study may be very useful for producing PHA from seaweeds containing polysaccharides such as starch. In addition, a 3.7-kbp DNA fragment containing the whole PHA synthase gene (*phaC*) was obtained from the strain UMI-21. The results of open reading frame (ORF) analysis suggested that the DNA fragment contained two ORFs, which were composed of 1,740 (*phaC*) and 564 bp (*phaR*). The deduced amino acid sequence of PhaC from strain UMI-21 shared high similarity with PhaC from *Ralstonia eutropha*, which is a representative PHA-producing bacterium with a class I PHA synthase. This is the first report for the cloning of the PHA synthase gene from *Massilia* species.

INTRODUCTION

Polyhydroxyalkanoates (PHAs), which are accumulated by numerous bacteria as carbon and energy storage materials under nutrient limitation and carbon excess, are aliphatic polyesters with thermoplasticity, optical activity, and biodegradability (1,2). More than 150 different monomers have been reported in PHAs (3). Based on the number of carbon atoms in their monomeric units, PHAs are classified into two groups, short-chain-length (scl) PHAs with C3 to C5 monomer units and medium-chain-length (mcl) PHAs with C6 to C14 monomer units (4). In general, scl PHAs exhibit thermoplastic properties and are mainly produced by *Ralstonia eutropha* (*R. eutropha*) strains, whereas mcl PHAs usually have elastomer- or adhesive-like properties and are synthesized by *Pseudomonas* species (5).

PHAs, which originate from renewable resources such as sugars and vegetable oils, have attracted great interest as alternatives to petrochemical-based plastics because of their material properties are similar to those of various thermoplastics and elastomers and their complete biodegradability (1,2). To date, several PHAs, such as poly(3-hydroxybutyrate) [P(3HB)], poly(3HB-co-3-hydroxyvalerate) [P(3HB-co-3HV)], and poly(3HB-co-3-hydroxyhexanoate) [P(3HB-co-3HHx)], have been commercially produced by microbial fermentation. However, a major issue regarding the industrial production of microbial PHAs is that their production cost is much higher than that of conventional petrochemical-based plastic materials. For industrial microbial production of PHAs, the cost of the fermentative substrate constitutes a significant proportion of the total cost (1,6,7). The cost of biopolymer production could be reduced by using inexpensive biomass, by-products, and waste streams as carbon sources, which can also help to convert these materials into value-added products. Accordingly, numerous research efforts have been made to exploit inexpensive fermentable raw materials as substrates for PHA production. For instance, we reported PHA

production from waste glycerol forming a biodiesel fuel production process by *Pseudomonas* sp. SG4502 (8). Gonzalez-Lopez et al. reported P(3HB) and P(3HB-co-3HV) production from alpechin, a wastewater from olive oil mills that is rich in sugars and phenolic compounds, by *Azotobacter chroococcum* H23 (9). Although various kinds of biomass have been used for material production by bacteria, most of them were obtained from land. Seaweed is an abundant biomass in the sea, and inedible seaweed exists in large quantities. In addition, seaweeds contain various kinds of polysaccharides such as alginic acid and starch, among others; therefore, they are a very attractive carbon source for material production by bacteria.

In this study, we attempted to screen for PHA-producing bacteria on *Ulva*, a green algae that contains starch. By using enrichment cultivation, we succeeded in isolating one microorganism, designated UMI-21, that can produce P(3HB) from starch as a sole carbon source. Based on the results of the 16S rDNA sequencing and phenotypic and phylogenetic analyses, strain UMI-21 was identified as *Massilia* sp. Furthermore, we succeeded in cloning a *pha* locus from the UMI-21 bacterium, and identified two open reading frames encoding PHA synthase (PhaC) and a repressor protein (PhaR). In addition to the production of PHA from various carbon sources, including glucose, maltose, maltotriose, and starch, the composition of the *phaC* gene cluster was also discussed in this article.

MATERIALS AND METHODS

Chemicals Peptone, yeast extract, casamino acids, glucose, corn starch, soluble starch, D-maltose, D-maltotriose, disodium hydrogen phosphate (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), ammonium chloride (NH_4Cl), and magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of reagent-grade quality or better.

1 **Media** Mineral salt (MS) medium, containing 3.6 g of Na₂HPO₄, 1.5 g of KH₂PO₄, 1.0 g of NH₄Cl,
2 0.2 g of MgSO₄·7H₂O, and 1 mL of trace element solution per liter of distilled water (10), was used
3 for the screening. Kato *et al.* reported that a higher CN ratio promoted PHA accumulation in
4 *Pseudomonas* sp. (10). Therefore, NH₄Cl was decreased to 0.5 g·L⁻¹ for PHA production.
5 Nutrient-rich (NR) medium containing 10 g of peptone, 2 g of yeast extract, and 10 g of meat extract
6 per liter of distilled water was used for precultivation of the bacteria. Modified MS (MSM) medium,
7 containing 3.6 g of Na₂HPO₄, 1.5 g of KH₂PO₄, 4.0 g of NH₄Cl, 1.2 g of MgSO₄·7H₂O, and 1 mL of
8 trace element solution per liter of distilled water, was used for cultivation in a jar fermenter. R2A
9 medium, containing 0.5 g of peptone, 0.5 g of yeast extract, 0.5 g of casamino acids, 0.5 g of glucose,
10 1.5 g of starch, 0.3 g of KHPO₄, 0.05 g of MgSO₄·7H₂O, 0.3 g of sodium pyruvate, and 15 g of agar
11 per liter of distilled water, was used for growth on plates.

12 **Isolation of bacteria accumulating polyhydroxyalkanoate** *Ulva*, a seaweed collected from the
13 coast, was used as a source of PHA-producing bacteria. Each seaweed sample was added to 50 mL of
14 MS medium containing 1% (w/v) starch and incubated for 3 days at 30°C with shaking at 150
15 strokes·min⁻¹. After repeating the subcultures several times, each culture was streaked on MS agar
16 medium containing 1% (w/v) starch and incubated at 30°C for 72 h. The isolated strains were tested
17 for PHA synthesis. The cells were inoculated in 5 mL of NR medium at 30°C for 16 h. Then, the
18 cultures were inoculated into 100 mL of nitrogen-limited MS medium containing 1% (w/v) starch in
19 500-mL Erlenmeyer flasks with baffles and incubated at 30°C for 72 h with shaking at 150 rpm. The
20 cells were collected by centrifugation at 10,000 × g for 10 min. The collected cells were washed twice
21 with distilled water and then lyophilized. The dried cell extracts were subjected to gas
22 chromatography (GC) analysis as described in the following paragraph.

23 **Production of polyhydroxyalkanoate** The isolated PHA-producing bacterium UMI-21 was cultured

1 in 5 mL of NR medium at 30°C for 24 h. The cultures were inoculated into 100 mL of
2 nitrogen-limited MS medium containing a carbon source (2% [w/v]) in 500-mL Erlenmeyer flasks
3 with baffles, and then incubated at 30°C with shaking at 150 rpm for 72 h. Corn starch, soluble starch,
4 glucose, maltose, and, maltotriose were used as carbon sources. Bacterial cells were collected by
5 centrifugation. The collected bacterial cells were washed twice with distilled water, and then
6 lyophilized. Polymer was extracted from the dried microbial cell bodies by using a Soxhlet extractor
7 with chloroform as a solvent, and then precipitated by the addition of methanol after the solvent was
8 removed with an evaporator. The cell dry weights, PHA yields, and PHA contents were described as
9 average values of three independent samples with standard deviations.

10 To evaluate the possibility of using UMI-21 for the large-scale production of PHA from
11 starch, the bacterium was cultivated in a 10-L jar fermenter (BMS-10NP3; ABLE & Biott Co., Ltd.,
12 Tokyo, Japan). First, UMI-21 was cultured in 250 mL of NR medium at 30°C for 24 h. Then, the
13 starter culture was inoculated into 5 L of MSM medium containing 5% (w/v) soluble starch in a 10-L
14 jar fermenter and incubated at 30°C with shaking at 150 rpm for 72 h. The carbon source
15 concentration and C/N ratio in the MSM medium for jar fermenter experiment was increased to
16 improve the cell growth and PHA content. The product was prepared as described above.

17 **Analyses** The isolated PHA-producing bacterium UMI-21 was cultivated on an R2A plate, and
18 genomic DNA was prepared from a single colony with PrepMan Ultra Reagent (Life Technologies
19 Japan, Tokyo, Japan). The PCR product was purified with the QIAquick Gel Extraction Kit (Qiagen
20 Japan, Tokyo, Japan), and then directly sequenced with the ABI PRISM 3100 Genetic Analyzer (Life
21 Technologies Japan). The strain UMI-21 16S rDNA sequence was determined by using BLAST
22 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the sequence was deposited in the DNA Data Bank of
23 Japan (DDBJ; accession number AB823088).

Polymer composition and accumulation were analyzed with the GC-2010 capillary GC system (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and a Zebron ZB-1 GC capillary column (0.25 mm i.d. × 30 m; Phenomenex, Inc., CA, USA). A sample was prepared as follows: approximately 10 mg of polymer or 50 mg of dried microbial cell was treated with a solution containing 1.7 ml of methanol, 0.3 ml of sulfuric acid and 2.0 ml of chloroform at 100°C for 3 h to convert the constituents to their methyl esters. One milliliter of water was added to the reaction mixture, and then, the lower chloroform layer was used for GC analysis.

The ¹H-NMR spectra of the polymers were obtained in deuterated chloroform (CDCl₃) using a Bruker MSL400 spectrometer (400 MHz) at a 90° pulse with a 4-ms, 3000-Hz spectral width and a 4-s repetition rate. The chemical shifts were reported in ppm with tetramethylsilane as the internal reference.

The molecular weights of the obtained polymers were determined by using a gel-permeation chromatography (GPC) system (Shimadzu Corporation, Kyoto, Japan) equipped with tandem TSKgel Super HZM-H columns (6.0 mm i.d. × 150 mm; Tosoh Corporation, Tokyo, Japan) at 40°C. Chloroform was used as an eluate, and the calibration was performed using polystyrene samples as standards.

Cloning and sequencing of the *pha* locus from UMI-21 Two primers, phaCF1_FW (5'-ATCAACAA(G/A)T(T/A)CTAC(A/G)TC(C/T)T(C/G)GACCT-3') and phaCR4_RV (5'-AGGTAGTTGT(T/C)GAC(C/G)(A/C)(A/C)(G/A)TAG(T/G)TCCA-3'), were used to obtain a partial DNA fragment of the PHA synthase gene from UMI-21 (11). DNA was amplified by PCR using UMI-21 genomic DNA as a template and KOD FX polymerase (Toyobo Co., Ltd., Osaka, Japan). The reaction conditions consisted of an initial 2-min denaturation step at 94°C, followed by 35 cycles of 98°C for 10 s, 50°C for 30 s, and 68°C for 1 min, with a final elongation step of 68°C for

5 min (12). The amplified 500-bp DNA fragment was purified with a gel extraction kit (Qiagen Japan). The nucleotide sequence of the fragment was determined with the ABI PRISM 3100 Genetic Analyzer. The upper (5'-region of *phaC* gene) and lower (3'-region of *phaC* and whole *phaR* genes) regions of the amplified 500-bp DNA fragment were cloned with the Takara LA PCR™ in vitro Cloning Kit (Takara Bio, Inc.) using cassettes and cassette-primers method.

RESULTS

Phenotypic and phylogenetic characterization of a bacterium producing PHA To obtain bacteria capable of producing PHA from a seaweed *Ulva*, we first performed enrichment cultivation in MS medium containing starch as a carbon source. *Ulva*, a seaweed collected from the coast, was used as a source of PHA-producing bacteria. The culture solution was spread onto an MS plate containing 1% (w/v) starch, and then incubated at 30°C for 72 h. The amount and monomer composition of the PHA accumulated in the bacterial cells were determined by GC analysis. As a result, we found a bacterium that could produce P(3HB) from starch and named the bacterium UMI-21.

We analyzed the microbiological properties of strain UMI-21, and found that strain UMI-21 is an aerobic, gram-negative, motile, non-spore-forming, rod-shaped bacillus (0.8–1.0 × 1.5–2.0 μm). The strain was able to grow at temperatures of up to 30°C and at pH 5.5–8.0 in MS medium containing 1% (w/v) starch. Anaerobic growth did not occur in the presence of nitrate. Tween-80, esculin, and starch were hydrolyzed by the bacterium, but urea, arginine, and gelatine were not hydrolyzed. Strain UMI-21 did not assimilate L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, potassium gluconate, *n*-capric acid, adipic acid, DL-malic acid, sodium citrate, or phenylacetic acid.

Analysis of the 16S rDNA sequence using BLAST indicated that strain UMI-21 belongs to the genus *Massilia* and that the closest phylogenetic neighbors of UMI-21 are *Massilia alkalitolerans*

(96.3% identity; accession number AY679161) and *Massilia varians* (97.8% identity; accession number AM774587). Phylogenetic analyses using Molecular Evolutionary Genetics Analysis (MEGA) software (ver. 4.0) showed that strain UMI-21 was clustered with other bacteria of the genus *Massilia* (Fig. 1). Based on the results of the 16S rDNA sequencing and phenotypic and phylogenetic analyses, strain UMI-21 was identified as *Massilia* sp. (13–18).

Polyhydroxyalkanoate productivity of strain UMI-21 To investigate the PHA productivity of strain UMI-21 from starch, the strain was cultivated under nitrogen-limiting conditions in MS medium supplemented with corn starch or soluble starch as a carbon source. The polymers that accumulated in the cell were extracted from dried cells with chloroform and were analyzed. When corn and soluble starch were used as the carbon sources, strain UMI-21 reached total biomasses of $3.95 \pm 0.32 \text{ g} \cdot \text{L}^{-1}$ and $3.31 \pm 0.03 \text{ g} \cdot \text{L}^{-1}$, respectively (Table 1). The PHA content in the cells was 30.3% (w/w) and 27.2% (w/w), respectively (Table 1). The weight-average molecular weight (M_w) and polydispersity (M_w/M_n) of the products made from corn starch were 7.6×10^5 and 2.8, respectively (Table 1). The ^1H NMR and GC analyses clearly indicated that the product was P(3HB) (Fig. 2).

Starch is a high molecular weight homopolymer of glucose. In general, bacteria incorporate monosaccharides and/or oligosaccharides generated by starch-hydrolysing enzymes such as amylase and gluco-amylase. To confirm the assimilation mechanism, we analyzed PHA production by UMI-21 using glucose, maltose, and maltotriose as carbon sources. When maltose and maltotriose were used as carbon sources, the same results were observed as those using starch. In contrast, both cell growth and PHA production were significantly lower when glucose was used as a carbon source than when starch was used. These results strongly suggest that strain UMI-21 degrades starch and incorporates the resulting oligosaccharides such as maltose and maltotriose. Therefore, strain UMI-21 could have a specific incorporation mechanism for maltooligosaccharides. Their molecular

1 weights and polydispersities were $7.6\text{--}9.5 \times 10^5$ and 2.6–2.9, respectively, and they were comparable
2 to each other.

3 **Polyhydroxyalkanoate production using a jar fermenter** To evaluate the possibility of using
4 UMI-21 for the large-scale production of PHA from starch, the bacterium was cultivated in a 10-L jar
5 fermenter. The result of the ^1H -NMR analysis suggested that the polymer produced by the cultivation
6 of UMI-21 using a jar fermenter was P(3HB). The total biomass (cell dry weight) and PHA content
7 were $8.29 \text{ g}\cdot\text{L}^{-1}$ and 45.5% (w/w), respectively. These values were higher than those obtained after
8 cultivation in a flask, suggesting the possibility of large-scale PHA production by UMI-21 from
9 starch.

10 **Cloning and sequencing of DNA fragments containing the PHA synthase gene (*phaC*)** The PHA
11 synthesis genes of these bacteria have not yet been identified, though PHA production from starch by
12 *Massilia* species has been reported (19). We attempted to clone the PHA synthesis genes of UMI-21
13 as follows. First, approximately 500-bp DNA fragment was amplified by PCR using primers
14 *phaCF1_FW* and *phaCR4_RV*, which were designed based on the consensus sequence of *phaC* (11).
15 The nucleotide sequence of the fragment was determined with the ABI PRISM 3100 Genetic
16 Analyzer and was subjected to BLAST analysis. The BLAST results indicated that the fragment was
17 a part of a PHA synthase gene. The Takara LA PCR™ in vitro Cloning Kit was used to clone a gene
18 fragment containing the whole *phaC* gene and surrounding regions. Finally, a 3.7-kbp DNA fragment
19 containing the whole *phaC* gene was obtained (Fig. 3). The nucleotide sequence of the fragment was
20 determined by primer walking (Fig. 3). The results of open reading frame (ORF) analysis suggested
21 that the DNA fragment contained two ORFs, which were composed of 1,740 and 564 bp (DDBJ
22 accession no. AB823089). For *phaC* gene, the putative promoter regions are underlined and the
23 putative ribosomal binding site is boxed (Fig. 3-b).

A database search with the ORFs using Protein BLAST showed that the ORFs shared sequence similarity with PhaC and PhaR (Table 2). PHA synthases are divided into four classes (classes I to IV) based on their activities toward substrates with different carbon chain lengths and their subunit compositions (4). The deduced amino acid sequence of PhaC from strain UMI-21 shared high similarity with PhaC from *R. eutropha*, which is a representative PHA-producing bacterium with a class I PHA synthase (Table 2).

Figure 4 shows partial sequence alignments of the PHA synthases (PhaCs) from strain UMI-21, *Janthinobacterium* sp. HH01, *Herbaspirillum seropedicae*, *R. eutropha* H16, *Variovorax paradoxus*, and *Hydrogenophaga* sp. PBC. It has been reported that all PHA synthases contain a putative lipase box, [Gly-Xaa-(Ser/Cys)-Xaa-Gly-Gly], in which the essential active site serine is replaced with a cysteine in the PHA synthase (3,20). The PhaC from strain UMI-21 also contained a putative lipase box with a cysteine residue (Fig. 4). Furthermore, Rehm *et al.* reported that the highly conserved Cys-319, Asp-480, and His-508 residues in PhaC from *R. eutropha* constituted the catalytic triad (20). Since these amino acids are conserved in the sequences deduced from *phaC* in strain UMI-21 (Fig. 4: Cys-315, Asp-461, and His-493), they may constitute a catalytic triad.

DISCUSSION

We successfully isolated a novel bacterium, strain UMI-21, that is able to accumulate PHA from starch. Taxonomic studies and 16S rDNA gene-sequence analysis revealed that strain UMI-21 was phylogenetically related to species of the genus *Massilia* (13–18). The closest species is *M. varians* CCUG 35299; PHA productivity has not been reported for this species. Moreover, strain UMI-21 could not assimilate L-arabinose, D-mannose, D-mannitol, N-acetyl-D-glucosamine, potassium gluconate, n-capric acid, adipic acid, DL-malic acid, sodium citrate, and phenyl acetate. As a result, we concluded that strain UMI-21 was a *Massilia* sp.

1 *Massilia timonae* was for the first time isolated and identified from the blood of an
2 immunocompromised patient with cerebellar lesions by La Scola et al. (13). The genus *Massilia*
3 belongs to the family *Oxalobacteraceae*, and 18 species of this genus have been isolated from the
4 various environmental conditions such as air, soil, and water. Recently, Cerrone *et al.* reported that
5 bacteria in the genus *Massilia* can accumulate PHA from starch, and *M. albidiflava*, *M. lutea*, *M.*
6 *dura*, *M. plicata*, *M. brevitalea*, *M. aerilata*, and *M. aurea* were used in their experiment (19). Some
7 strains could produce PHA, and higher PHA productivity was achieved when starch was used as the
8 carbon source in the growth medium instead of glucose (19). These results are identical to those we
9 observed in our experiment with UMI-21. In general, bacteria incorporate monosaccharides and/or
10 oligosaccharides generated by hydrolysis with starch-hydrolysing enzymes such as amylase and
11 gluco-amylase because starch is a high molecular weight polymer composed of glucose.
12 Interestingly, more PHA was obtained when starch was used as a carbon source. Therefore, to
13 estimate the mechanism underlying the assimilation of starch in strain UMI-21, we measured PHA
14 production from glucose, maltose, and maltotriose. When maltose or maltotriose was used as a carbon
15 source, PHA content increased up to 40% (w/w), suggesting that oligosaccharides are preferably
16 ingested by the bacterium. In contrast, since cells did not grow primarily when glucose was used as a
17 carbon source (Table 1), glucose was not ingested by the bacterium. In *Saccharomyces cerevisiae*, the
18 Agt1 transporter has relatively high activity towards maltotriose and maltose (21), which is related to
19 the uptake of maltose and maltotriose by the cells. A transporter specific for cyclodextrins (CDs),
20 which are produced from maltodextrins by CD-glucanotransferases (CGTases), has been reported for
21 bacteria such as *Klebsiella oxytoca* (22,23). Although the detailed metabolic mechanism of starch
22 hydrolysis in strain UMI-21 is yet unknown, UMI-21 may have an uptake mechanism for maltose and
23 maltotriose. Since glucose is a common carbon source for most microorganisms, these unique

1 substrate-uptake mechanisms for starch metabolism could be a strategy of winning competitors.
2 Further studies are needed to determine the unique metabolic system for starch utilization in UMI-21.
3 The nucleotide sequence of more than 50 PHA synthase genes from a wide variety of
4 bacteria has been determined. PHA synthases are classified into four classes according to their
5 primary structures deduced from these sequences, substrate specificities, and subunit composition (4).
6 Class I and class II PHA synthases comprise enzymes consisting of only one type of subunit (PhaC).
7 Class III PHA synthases comprise enzymes consisting of two different types of subunits, PhaC and
8 PhaE. Class IV PHA synthases comprise enzymes consisting of two different types of subunits. Based
9 on the result that strain UMI-21 produced P(3HB) and the deduced amino acid sequence of PhaC
10 shared similarity with PhaC from *R. eutropha*, which is a representative PHA-producing bacterium
11 with a class I PHA synthase, we concluded that UMI-21 PhaC belonged to class I.

12 PHA synthase genes and other genes related to the metabolism of PHA are often clustered
13 in bacterial genomes. In *R. eutropha*, the genes encoding the class I PHA synthase (*phaC*),
14 β -ketothiolase (*phaA*), and NADP-dependent acetoacetyl-CoA reductase (*phaB*) constitute the
15 *phaCAB* operon (24). In UMI-21, *phaR* is downstream of *phaC* instead of *phaA* and *phaB*. Very
16 recently, the sequence of *M. timonae* CCUG 45783 (accession No. NZ_JH992933) has been
17 determined. Although *phaR* was downstream of *phaC*, as in strain UMI-21, other genes related to
18 PHA synthesis, such as *phaA* and *phaB*, were not found near *phaC* and *phaR*. In *R. eutropha*, PhaR is
19 proposed to function as a repressor of *phaP* and *phaR* transcription under conditions in which P(3HB)
20 does not accumulate (25). Once the accumulation of P(3HB) is initiated, PhaR is thought to dissociate
21 from the promoter and bind to oligomeric or polymeric forms of (HB)_n. The PhaR proteins from *R.*
22 *eutropha* and strain UMI-21 are approximately 80% similar. Therefore, strain UMI-21 PhaR could
23 have a function similar to that of *R. eutropha* PhaR (26), though the existence of *phaP* genes have not

REFERENCES

1. **Chen, G.Q.:** A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Rev **38**, 2434–2446 (2009).
2. **Sudesh, K., Abe, H. and Doi, Y.:** Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci **25**, 1503–1555 (2000).
3. **Steinbüchel, A. and Valentin, H.E.:** Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett **128**, 219–228 (1995).
4. **Rehm, B.H.A.:** Polyester syntheses: natural catalysts for plastics. Biochem J **376**, 15–33 (2003).
5. **Van der Walle, G.A.M., de Koning, G.J.M., Weusthuis, R.A. and Eggink, G.:** Properties, modifications and applications of biopolyesters. Adv Biochem Eng Biotechnol **71**, 263–291 (2001).
6. **Choi, J. and Lee, S.Y.:** Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation. Bioprocess Eng **17**, 335–342 (1997).
7. **Choi, J. and Lee, S.Y.:** Factors affecting the economics of polyhydroxyalkanoates production by bacterial fermentation. Appl Microbiol Biotechnol **51**, 13–21 (1999).
8. **Satoh, Y., Tajima, K., Han, X., Matsushima, T., Ohshima, T., Kawano, S., Erata, T., Dairi, T. and Munekata, M.:** Isolation of a thermotolerant bacterium producing medium-chain-length polyhydroxyalkanoate. J Appl Microbiol **111**, 811–817 (2011).
9. **Gonzalez-Lopez, J., Pozo, C., Martinez-Toledo, M.V., Rodelas, B. and Salmeron, V.:** Production of polyhydroxyalkanoates by *Azotobacter chroococcum* H23 in wastewater from olive oil mills (alpechin). Int Biodeterior Biodegrad **38**, 271–276 (1996).
10. **Kato, M., Bao, H.J., Kang, C.K., Fukui, T. and Doi, Y.:** Production of a novel copolyester of

3-hydroxybutyric acid and medium chain length 3-hydroxyalkanoic acid by *Pseudomonas* sp.
61-3 from sugars. Appl Microbiol Biotechnol **45**, 363–370 (1996).

11. **Sheu, D.-S., Wang, Y.-T. and Lee C.-Y.:** Rapid detection of
polyhydroxyalkanoate-accumulating bacteria isolated from the environment by colony PCR.
Microbiology **146**, 2019–2025 (2000).

12. **Tajima, K., Han, X., Satoh, Y., Ishii, A., Araki, Y., Munekata, M. and Taguchi, S.:** In vitro
synthesis of polyhydroxyalkanoate (PHA) incorporating lactate (LA) with a block sequence by
using a newly engineered thermostable PHA synthase from *Pseudomonas* sp. SG4502 with
acquired LA-polymerizing activity. Appl Microbiol Biotechnol **94**, 365–376 (2012).

13. **La Scola, B., Birtles, R.J., Mallet, M.N. and Raoult, D.:** *Massilia timonae* gen. nov., sp. nov.,
isolated from blood of an immunocompromised patient with cerebellar lesions. J Clin Microbiol **36**,
2847–2852 (1998).

14. **Busse, H.J., Hauser, E. and Kämpfer, P.:** Description of two novel species, *Sphingomonas*
abaci sp. nov. and *Sphingomonas panni* sp. nov. Int J Syst Evol Microbiol **55**, 2565–2569
(2005).

15. **Kämpfer, P., Falsen, E. and Busse, H.J.:** *Naxibacter varians* sp. nov. and *Naxibacter*
haematophilus sp. nov., and emended description of the genus *Naxibacter*. Int J Syst Evol
Microbiol **58**, 1680–1684 (2008).

16. **Kämpfer, P., Lodders, N., Martin, K. and Falsen, E.:** Revision of the genus *Massilia* La
Scola et al. 2000, with an emended description of the genus and the inclusion of all species of
the genus *Naxibacter* as new combinations, and a proposal of *Massilia consociate* sp. nov. Int J
Syst Evol Microbiol **61**, 1528–1533 (2011).

17. **Weon, H.Y., Kim, B.Y., Son, J.A., Jang, H.B., Hong, S.K., Go, S.J. and Kwon, S.W.:**

- Massilia aerilata* sp. nov., isolated from an air sample. Int J Syst Evol Microbiol **58**, 1422–1425 (2008).
18. **Wang, J., Zhang, J., Pang, H., Zhang, Y., Li, Y. and Fan, J.:** *Massilia flava* sp. nov., isolated from soil. Int J Syst Evol Microbiol **62**, 580–585 (2012).
19. **Cerrone, F., Sánchez-Peinado, M., Rodríguez-Díaz, M., González-López, J. and Pozo, C.:** PHA production by strains belonging to *Massilia* genus from starch. Starch/Stärke **63**, 236–240 (2011).
20. **Rehm, B.H.A., Antonio, R.V., Spiekermann, P., Amara, A.A. and Steinbüchel, A.:** Molecular characterization of the poly(3-hydroxybutyrate) (PHB) synthase from *Ralstonia eutropha*: in vitro evolution, site-specific mutagenesis and development of a PHB synthase protein model. Biochim Biophys Acta **1594**, 178–190 (2002).
21. **Vigren, V., Multanen, J-P., Ruohonen, L. and Londesborough, J.:** The temperature dependence of maltose transport in ale and lager strains of brewer's yeast. FEMS Yeast Res **10**, 402–411 (2010).
22. **Feederle, R., Pajatsch, M., Kremmer, E. and Böck, A.:** Metabolism of cyclodextrins by *Klebsiella oxytoca* m5a1: purification and characterisation of a cytoplasmically located cyclodextrinase. Arch Microbiol **165**, 206–212 (1996)
23. **Pajatsch, M., Gerhart, M., Peist, R., Horlacher, R., Boos, W. and Böck, A.:** The periplasmic cyclodextrin binding protein CymE from *Klebsiella oxytoca* and its role in maltodextrin and cyclodextrin transport. J Bacteriol **180**, 2630–2635 (1998).
24. **Peoples, O.P. and Sinskey, A.J.:** Poly- β -hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. J Biol Chem **264**, 15298–15303 (1989).
25. **Pötter, M., Madkour M.H., Mayer, F. and Steinbüchel, A.:** Regulation of phasin expression

and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. Microbiology **148**, 2413–2426 (2002).

26. **York, G.M., Stubbe, J. and Sinskey, A.J.:** The *Ralstonia eutropha* PhaR protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. J Bacteriol **184**, 59–66 (2002).

27. **Gonzalez-Garcia, Y., Rosales, M.A., Gonzalez-Reynoso, O., Sanjuan-Duenas, R. and Cordova, J.:** Polyhydroxybutyrate production by *Saccharophagus degradans* using raw starch as carbon source. Eng Life Sci **11**, 59–64 (2011).

28. **Halami, P.M.:** Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. World J Microbiol Biotechnol **24**, 805–812 (2008).

29. **Chien, C.C. and Ho, L.Y.:** Polyhydroxyalkanoates production from carbohydrates by a genetic recombinant *Aeromonas* sp. Lett Appl Microbiol **47**, 587–593 (2008).

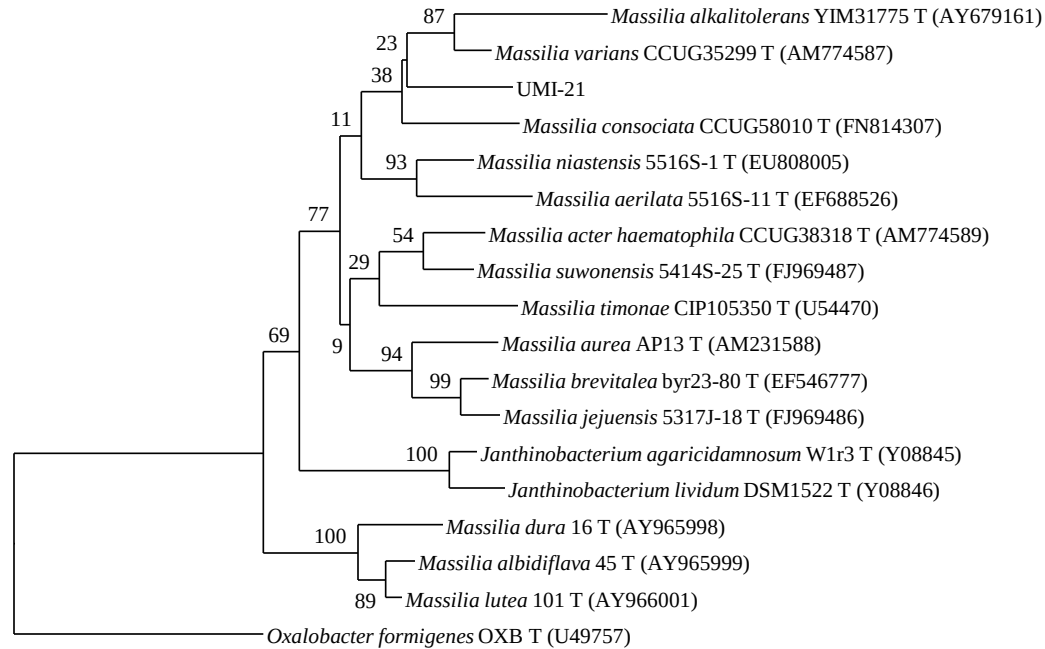
30. **Haas, R., Jin, B. and Zepf, F.T.:** Production of poly(3-hydroxybutyrate) from waste potato starch. Biosci Biotechnol Biochem **72**, 253–256 (2008).

31. **Huang, T.Y., Duan, K.J., Huang, S.Y. and Chen, C.W.:** Production of polyhydroxyalkanoates from inexpensive extruded rice bran and starch by *Haloferax mediterranei*. J Ind Microbiol Biotechnol **33**, 701–706 (2006).

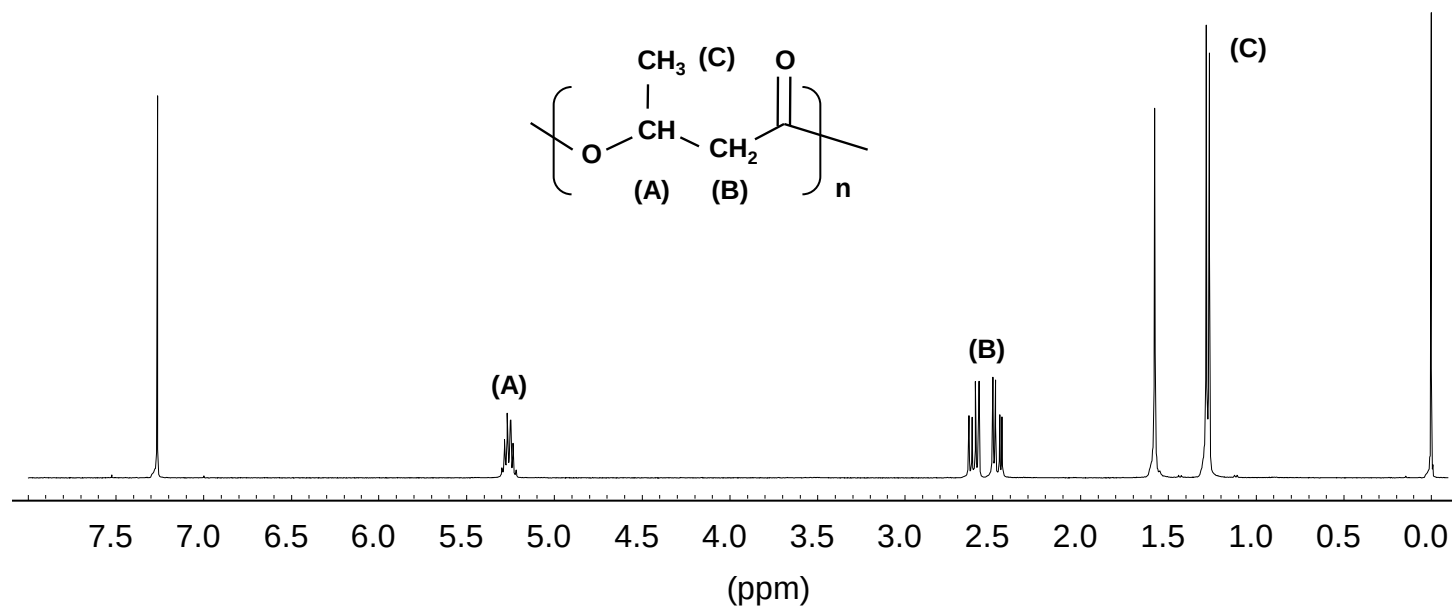
32. **Sheu, D.S., Chen, W.M., Yang, J. Y. and Chang, R.C.:** Thermophilic bacterium *Caldimonas taiwanensis* produces poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from starch and valerate as carbon sources. Enzyme Microb Technol **44**, 289–294 (2009).

Figure and Table Legends

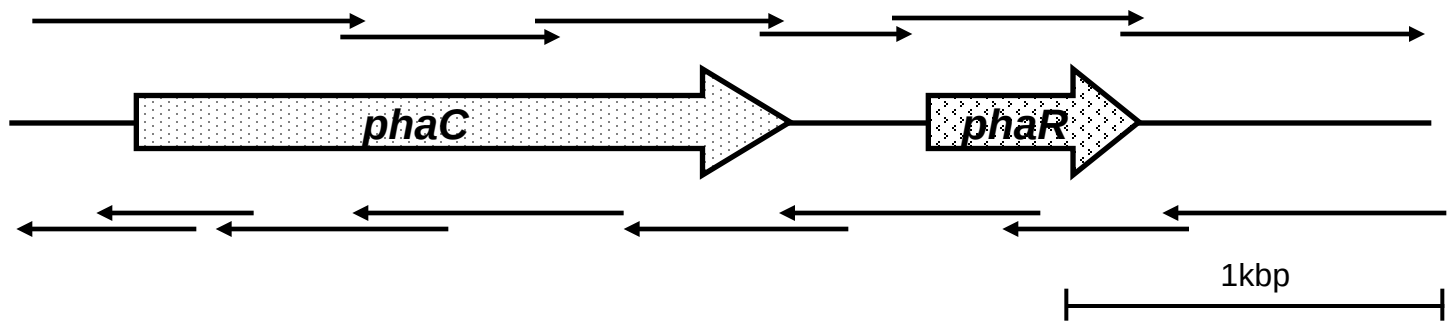
- Fig. 1** Phylogenetic tree of strain UMI-21 and related microorganisms. The tree is based on an alignment of the 16S rDNA genes of UMI-21 and type strains in the genera *Massilia* (14 strains), *Janthinobacterium* (2 strains), and *Oxalobacter* (1 strain).
- Fig. 2** ^1H -NMR spectrum of the polymer produced by UMI-21 from corn starch.
- Fig. 3** (a) Organization of the UMI-21 PHA biosynthesis genes (*phaC* and *phaR*) and the DNA sequencing strategy and (b) the putative promoter regions and ribosomal binding site for *phaC* gene. The arrows show the primers and the sequenced regions. The putative promoter regions are underlined and the putative ribosomal binding site is boxed.
- Fig. 4** Alignment of the partial deduced amino acid sequences of PHA synthases from strain UMI-21 and similar proteins from *Janthinobacterium* sp. HH01, *Herbaspirillum seropedicae*, *Ralstonia eutropha* H16, *Variovorax paradoxus*, *Hydrogenophaga* sp. PBC. The portions of the PHA synthases thought to comprise the catalytic domain, including the putative catalytic triad (C-D-H), are shown.
- Table 1** Cell dry weights (CDWs), PHA yields, molecular weights, polydispersities, PHA contents, and monomer compositions of PHAs produced by UMI-21.
- Table 2** Homology of the PHA synthesis gene products of strain UMI-21 to proteins of other bacteria.



0.01



a



b CGGCGACGATCCACATGCTTTTCTCCAAGGTTGTAGACCCCGATTCTAAACAACGGATGCTGACATGAACATGCCT
M N M P

Putative lipase box

	315						461		493							
UMI-21	---	G	F	C	V	G	G	---	E	D	H	---	G	H	I	---
<i>Janthinobacterium</i> sp. HH01	---	G	F	C	V	G	G	---	E	D	H	---	G	H	I	---
<i>Herbaspirillum seropedicae</i>	---	G	F	C	V	G	G	---	D	D	H	---	G	H	I	---
<i>Ralstonia eutropha</i> H16	---	G	F	C	V	G	G	---	E	D	H	---	G	H	I	---
<i>Variovorax paradoxus</i>	---	G	F	C	V	G	G	---	E	D	H	---	G	H	I	---
<i>Hydrogenophaga</i> sp. PBC	---	G	F	C	V	G	G	---	E	D	H	---	G	H	I	---

Table 1 Cell dry weights (CDWs), PHA yields, molecular weights, polydispersities, PHA contents, and monomer compositions of PHAs produced by UMI-21.

Carbon sources (2%)	CDW ^a (g/l)	PHA yield (g/l)	$M_w \times 10^{5b}$	M_w / M_n	PHA [% (w/w)%]	Momomer composition ^c (mol%)
						3HB
Corn starch	3.95±0.32	1.20±0.04	7.6	2.8	30.3±0.17	100
Soluble starch	3.31±0.03	0.90±0.06	8.7	2.9	27.2±1.66	100
Glucose	0.67±0.04	0.02±0.01	7.9	3.7	2.4±1.6	100
Maltose	3.68±0.08	1.39±0.08	7.6	2.6	37.7±1.31	100
Maltotriose	3.75±0.17	1.51±0.06	9.5	2.7	40.2±2.06	100

^aCell dry weight (CDW) was defined as cell components plus PHA.

^bMolecular weights were determined by gel permeation chromatography (GPC).

^cMonomer compositions were determined by ¹H-NMR and GC analyses.

Table 2 Homology of the PHA synthesis gene products of strain UMI-21 to proteins of other bacteria.

Organism	PhaC		PhaR	
	accession No.	identity (%)	accession No.	identity (%)
<i>Janthinobacterium</i> sp. HH01	WP_008447857.1	69	WP_008447858.1	87
<i>Herbaspirillum seropedicae</i>	YP_003776395.1	62	WP_017451769.1	80
<i>Ralstonia eutropha</i> H16	YP_725940.1	62	YP_725943.1	77
<i>Variovorax paradoxus</i>	WP_018902870.1	60	WP_018905029.1	62
<i>Hydrogenophaga</i> sp. PBC	WP_009515239.1	56	WP_009517543.1	61